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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEMS FOR THE SEPARATION OF BENZODIAZEPINES AND THEIR METABOLITES

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SUMMARY

The high-performance liquid chromatographic (HPLC) retention characteristics of 21 benzodiazepine drugs and some of their metabolites have been examined on both silica and ODS-silica packing materials. Four HPLC systems have been considered and retention data are presented for the drugs on these systems. The correlation of retention data on the systems is considered with reference to the problem of identifying unknown benzodiazepines.

INTRODUCTION

The transfer of high-performance liquid chromatography (HPLC) methods between laboratories has often been hindered by the lack of any column standardization. It has long been recognized that packing materials of the same type (*e.g.*, silica, ODS-silica) but from different manufacturers can have very different chromatographic properties. Consequently much time is often wasted in the modification of eluents taken from published procedures in order to achieve the appropriate separations on the commercial brand of packing material available in the laboratory. Furthermore, when a laboratory is engaged in many different HPLC assays any attempt to stock a comprehensive range of commercial products is impractical and prohibitively expensive. For example, there are over 30 different brands of both silica and ODS-silica packing materials available[1]. These considerations have led forensic science laboratories in the U.K. to standardize on the HPLC packing materials used for routine work. Experience has shown that most separations can be achieved using silica or ODS-silica and common commercial products of these types are now being used in 11 laboratories throughout the U.K. This practical decision has greatly facilitated the transfer of HPLC methods between laboratories while conveniently reducing the total number of materials which need to be stocked.

Further work has involved the development of HPLC eluents to use with the standardized packing materials suitable for use in forensic drug analysis. As part of this work systems have been published for the separation of various drug groups along with tables of appropriate retention data (barbiturates[2,3], amphetamines[4,5],

narcotic analgesics[5], local anaesthetics[6] and ergot alkaloids[7]). In a similar way large collections of thin-layer chromatography[8] and gas–liquid chromatography[9] retention data have been collected to aid in the identification of drugs.

HPLC has been widely used for the analysis of benzodiazepine drugs in pharmaceuticals and biological fluids, and recent reviews[10,11] give some indication of the range of different systems which have been adopted. Most papers have only considered a small number of compounds and no comprehensive data base of retention properties exists for these drugs. As well as systems for the parent drugs there are reports of systems suitable for metabolites and also for the benzophenones which arise from hydrolysis of the parent compounds[12]. Benzodiazepines are of considerable importance in forensic toxicology having hypnotic, tranquillising and/or anticonvulsant properties and thus they are often encountered in casework involving road traffic offences or drug overdose. Furthermore the range of benzodiazepines available has expanded rapidly over the last five years. The present paper presents HPLC systems suitable for the separation of benzodiazepines and their metabolites using the silica and ODS-silica packing materials adopted by forensic science laboratories in the U.K. Retention data for 21 drugs are presented including all benzodiazepines available for prescription in the U.K. in September 1983. Since that time one further drug, loprazolam, has become available.

EXPERIMENTAL

Materials

Methanol (HPLC grade) was obtained from Rathburn Chemicals (Walkerburn, U.K.). Perchloric acid (72%, AristaR) and trifluoroacetic acid (Spectrosol) were obtained from BDH (Poole, U.K.). Water was distilled in glass in the laboratory. All other chemicals used were of analytical grade.

The benzodiazepines and metabolites were from the drug collection of the Central Research Establishment, Home Office Forensic Science Service, except for desoxychlordiazepoxide[13]. This compound was prepared by refluxing chlordiazepoxide in phosphorus trichloride, boiling off the excess reagent and recrystallising the residue ($\times 2$) from ethanol giving the hydrochloride salt. The UV spectroscopic and mass spectrometric data for this product agreed with that already published[13,14].

The HPLC packing materials used were 5-μm Spherisorb S5W (Phase Separations, Queensferry, U.K.) and 5-μm ODS-Hypersil (Shandon, Runcorn, U.K.).

Apparatus

HPLC was performed with a Waters M6000A pump, a Rheodyne 7120 injection valve (fitted with a 20-μl loop) and a Perkin-Elmer LC75 variable-wavelength UV detector operated at 240 nm. The stainless-steel columns of 20 and 25 cm length (5 mm I.D.) were from Shandon and were packed using conventional slurry procedures. The ODS-silica packing material was dispersed in isopropanol with hexane as the pressurising solvent while the silica material was dispersed in methanol and pressurised with methanol.

Chromatography

Two eluents were necessary to accommodate the wide range of polarities en-

countered for the benzodiazepines on ODS-silica. The 55% (v/v) methanol eluent for system A was prepared by mixing methanol (550 ml), water (250 ml) and phosphate buffer (200 ml). The 70% (v/v) methanol eluent for system B was prepared by mixing methanol (700 ml), water (100 ml) and phosphate buffer (200 ml). The phosphate buffer (0.1 *M*) was prepared by dissolving sodium dihydrogen phosphate dihydrate (14.35 g, 0.092 moles) and disodium hydrogen phosphate (1.14 g, 0.008 moles) in water (1000 ml). The 55% (v/v) and 70% (v/v) eluents were found to have pH values of 7.25 and 7.67 respectively. A 20-cm ODS-Hypersil column was used with these eluents with flow-rates of 1.5 ml/min.

The perchloric acid eluent used for the chromatography of the benzodiazepines on a 25-cm Spherisorb column (system C) was prepared by adding perchloric acid (72%, 100 μl) to methanol (1000 ml). A flow-rate of 2 ml/min was used.

The trifluoroacetic acid eluent used for the chromatography of the drugs on a 25-cm silica column (system D) was prepared by mixing methanol (997 ml), water (2 ml) and trifluoroacetic acid (1 ml). A flow-rate of 2 ml/min was used with this eluent.

Drug samples were dissolved in 50% (v/v) aqueous methanol for injection onto the ODS-silica HPLC systems or in methanol for the silica HPLC systems. Retention data are expressed as capacity ratios, k', which are defined by $k' = (t_R - t_0)/t_0$, where t_R and t_0 are the retention times of the substance under investigation and a non-retained compound respectively.

RESULTS AND DISCUSSION

Previous work from this laboratory[15] has demonstrated the use of HPLC systems using ODS-silica columns for the analysis of benzodiazepines in forensic toxicology. In agreement with earlier observations it was found that a single isocratic eluent was not suitable for all benzodiazepines because of their widely varying polarities. Finally, two isocratic systems (A and B) were selected with eluents containing 55% and 70% (v/v) methanol, respectively along with a phosphate buffer. Table I shows the retention data (k' values) for the drugs and metabolites on the two systems listed in order of elution. The drug names used in this table are those given in ref. 16 wherever appropriate.

System A is more suitable for the separation of the more polar compounds while system B is needed for the hydrophobic benzodiazepines. Three compounds (diazepam, midazolam and ketazolam) having k' values greater than 8 with system A were also run on system B and it is interesting to note the inversion of elution order of diazepam and midazolam. After the compilation of the retention data, those compounds having similar k' values were co-chromatographed to give some information as to which could not be separated. This information is also included in the final column of Table I; it should only be treated as a guide since the resolution of the drugs will depend on the efficiency of the HPLC column used. With system A there are two clusters of compounds with k' values around 3 and 4.5 respectively where chromatographic resolution is poor.

Figs. 1 and 2 show chromatograms demonstrating the separation of the benzodiazepines and metabolites on the ODS-silica column with the 55% and 70% methanolic eluents. It can be seen that most compounds showed satisfactory peak shapes

TABLE I

HPLC RETENTION DATA FOR THE BENZODIAZEPINES AND METABOLITES ON ODS-SILICA (SYSTEMS A AND B)

Column: ODS-Hypersil, 5 μm (200 × 5 mm I.D.). System A eluent, methanol–water–phosphate buffer (0.1 *M*) (55:25:20, v/v/v); system B eluent, methanol–water–phosphate buffer (0.1 *M*) (70:10:20, v/v/v).

Elution order	*Compound*	*Capacity ratio (k')*	*Co-eluting** compounds*
System A			
1	7-Aminonitrazepam	0.46	
2	7-Acetamidonitrazepam	0.68	
3	Clorazepic acid	1.17	
4	Bromazepam	2.32	5
5	Demoxepam	2.42	4
6	Clonazepam	2.85	7, 8
7	Nitrazepam	2.96	6, 8
8	Desmethylclobazam	3.06	6, 7, 9
9	Flunitrazepam	3.15	8
10	Clobazam	3.91	
11	N-1-Hydroxyethylflurazepam	4.27	12, 13, 14, 15
12	Triazolam	4.38	11, 13, 14, 15
13	Desmethylchlordiazepoxide	4.47	11, 12, 14, 15
14	Lorazepam	4.60	11, 12, 13, 15, 16
15	Oxazepam	4.62	11, 12, 13, 14, 16
16	Alprazolam	4.70	14, 15
17	N-1-Desalkylflurazepam	5.19	
18	Temazepam	5.68	
19	Lormetazepam	6.32	20
20	Chlordiazepoxide	6.41	19
21	Nordazepam	8.00	
22	Diazepam	9.47	
23	Midazolam	9.75	
24	Ketazolam	12.81	
System B			
1	Midazolam	2.10	
2	Diazepam	2.29	
3	Ketazolam	2.45	
4	Flurazepam	3.19*	
5	Desoxychlordiazepoxide	3.85	
6	Prazepam	4.60	
7	Medazepam	7.05*	

* Tailing peak.

** Co-eluting compounds represented by their numbers in the elution order of the appropriate system.

with the exception of flurazepam and to a lesser extent medazepam which showed tailing peaks (Fig. 2).

The retention properties of the benzodiazepines have also been measured on a silica HPLC system involving an eluent of methanol containing 0.01% (v/v) perchloric acid (system C). HPLC systems of this type have been used for the analysis of several drugs, including some benzodiazepines, by Flanagan *et al.*[17–19] and Kelly

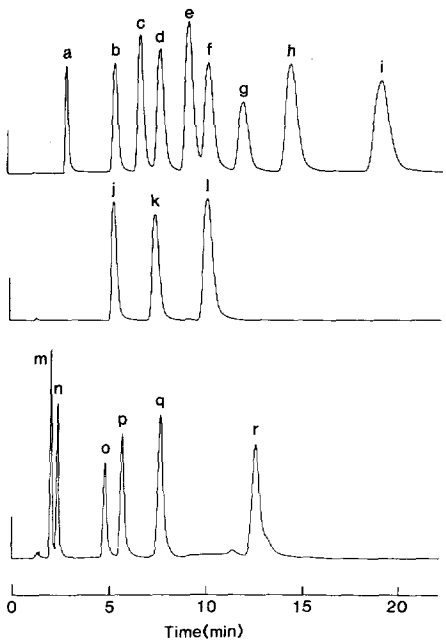

Fig. 1. Chromatography of benzodiazepines and metabolites on ODS-silica (system A). Column, ODS-Hypersil, 5 μm (200 × 5 mm I.D.); eluent, 55% methanol containing phosphate buffer (pH 7.25); flow-rate, 1.5 ml/min; detection, 240 nm. Peaks: a = clorazepic acid; b = nitrazepam; c = clobazam; d = oxazepam; e = temazepam; f = chlordiazepoxide; g = nordazepam; h = diazepam; i = ketazolam; j = clonazepam; k = triazolam; l = lormetazepam; m = 7-aminonitrazepam; n = 7-acetamidonitrazepam; o = demoxepam; p = desmethylclobazam; q = desmethylchlordiazepoxide.

et al.[20]. In this case a single isocratic system was found to elute all compounds with reasonable retention times and Table II gives the retention data arranged in order of elution. All compounds eluted with $k' \leqslant 6.5$. As with the ODS-silica systems, the co-eluting peaks are also indicated in Table II. For this system clusters of compounds close to the solvent front ($k' \leqslant 0.14$) and with k' values from 1.4 to 2 were poorly

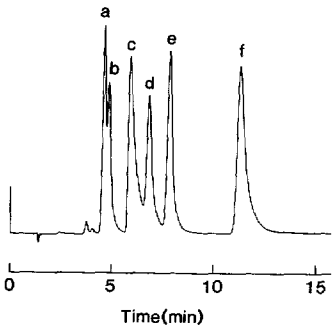

Fig. 2. Chromatography of benzodiazepines and metabolites on ODS-silica (system B). Column, ODS-Hypersil, 5-μm (200 × 5 mm I.D.); eluent, 70% methanol containing phosphate buffer (pH 7.67); flow-rate, 1.5 ml/min; detection, 240 nm. Peaks: a = diazepam; b = ketazolam; c = flurazepam; d = desoxychlordiazepoxide; e = prazepam; f = medazepam.

TABLE II

HPLC RETENTION DATA FOR THE BENZODIAZEPINES AND METABOLITES ON SILICA USING THE PERCHLORIC ACID ELUENT (SYSTEM C)

Column: Spherisorb S5W, 5 μm (250 × 5 mm I.D.). Eluent, methanol (1000 ml) containing perchloric acid (100 μl).

Elution order	*Compound*	*Capacity ratio (k')*	*Co-eluting** compounds*
1	7-Aminonitrazepam	0	2, 3, 4, 5
2	Desmethylclobazam	0.01	1, 3, 4, 5
3	Clobazam	0.03	1, 2, 4, 5
4	Demoxepam	0.03	1, 2, 3, 5
5	Ketazolam	0.04	1, 2, 3, 4
6	Lormetazepam	0.08	7
7	Lorazepam	0.14	6
8	Clonazepam	0.35	
9	Flunitrazepam	0.47	
10	Temazepam	0.60	
11	Oxazepam	0.73	
12	N-1-Hydroxyethylflurazepam	1.43	13
13	Nitrazepam	1.49	12, 14
14	N-1-Desalkylflurazepam	1.52	13, 15
15	Triazolam	1.83	14
16	7-Acetamidonitrazepam	1.93	17
17	Nordazepam	1.99	16, 18
18	Clorazepic acid	2.00	17
19	Prazepam	2.19	
20	Desmethylchlordiazepoxide	2.39	
21	Diazepam	2.49	
22	Alprazolam	2.79	
23	Chlordiazepoxide	2.87	
24	Bromazepam	2.99	
25	Medazepam	4.44*	26
26	Desoxychlordiazepoxide	4.55*	25
27	Midazolam	5.90*	
28	Flurazepam	6.50*	

* Tailing peak.

** Each co-eluting compound is represented by its number in the elution order.

resolved. Peak shapes with system C were generally good (Fig. 3) although some of the more strongly retained compounds (medazepam, desoxychlordiazepoxide, midazolam and flurazepam) showed some peak tailing.

It is clear that neither the HPLC system on silica (system C) or the systems on ODS-silica (systems A and B) are capable of resolving all the benzodiazepines and metabolites. The combination of these systems for drug identification may prove to be useful. Fig. 4 shows a plot of capacity ratios for compounds on the two columns. The wide scatter of data points confirms that the mechanisms of separation on the two columns are very different. The separation of the benzodiazepines on the ODS-silica column is undoubtably related to their differences in hydrophobicity while the mechanism of separation on the silica column with the methanolic perchloric acid eluent is less certain[19]. Fig. 4 shows that a good discrimination can be achieved for most of the compounds by a combination of retention data from both columns.

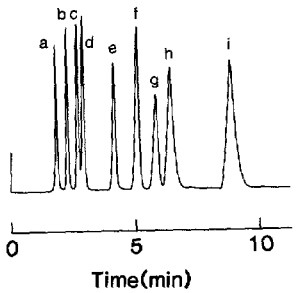

Fig. 3. Chromatography of benzodiazepines on silica using the perchloric acid eluent (system C). Column, Spherisorb S5W, 5 μm (250 × 5 mm I.D.); eluent, methanol (1000 ml) containing perchloric acid (100 μl); flow-rate, 2 ml/min; detection, 240 nm. Peaks: a = demoxepam; b = clonazepam; c = temazepam; d = oxazepam; e = nitrazepam; f = nordazepam; g = diazepam; h = chlordiazepoxide; i = medazepam.

As the mechanism of separation of the methanolic perchloric acid eluent on silica (system C) is poorly understood it is difficult to make systematic modifications to this system in order to separate unresolved compounds. A further eluent containing trifluoroacetic acid was tested on the silica column (system D) to investigate the effect of using an alternative acid to replace perchloric acid. Flanagan *et al.*[19] state that many strong acids (*e.g.*, sulphuric acid) can be used with this type of HPLC system but have not indicated whether changes in selectivity can be achieved by using different acids. Apparently, weak acids such as phosphoric acid are not suitable. The selection of trifluoroacetic acid was influenced by its low boiling point (72.4°C), since this should allow the easy removal of eluent from collected fractions. This could prove to be particularly useful for further confirmation of peak identity following HPLC analysis (*e.g.* mass spectrometry).

Table III shows the capacity ratios for the benzodiazepines and metabolites with the eluent containing trifluoroacetic acid on silica (system D) arranged in order to elution while Fig. 5 shows a chromatogram for eight compounds. As with the

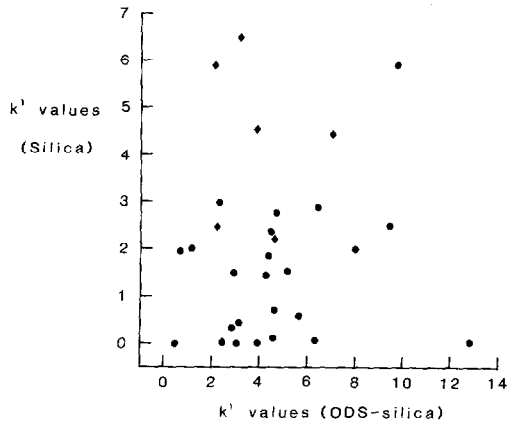

Fig. 4. Plot of capacity ratios for the benzodiazepines on silica using the perchloric acid eluent (system C) *versus* capacity ratios on ODS-silica (systems A and B). For details of the HPLC systems see Table IV. ● = System A (55% methanol); ◆ = system B (70% methanol).

TABLE III

HPLC RETENTION DATA FOR THE BENZODIAZEPINES AND METABOLITES ON SILICA USING THE TRIFLUOROACETIC ACID ELUENT (SYSTEM D)

Column: Spherisorb S5W, 5 μm (250 × 5 mm I.D.). Eluent, methanol–water–trifluoroacetic acid (997:2:1, v/v/v).

Compound	*Capacity ratio (k')*	*Compound*	*Capacity ratio (k')*
Ketazolam	0	Nitrazepam	1.60
7-Aminonitrazepam	0	Prazepam	2.42
Desmethylclobazam	0	Alprazolam	2.79
Clobazam	0.02	Clorazepic acid	3.12
Lormetazepam	0.02	Nordazepam	3.12
Lorazepam	0.05	Diazepam	3.35
Demoxepam	0.07	7-Acetamidonitrazepam	3.58
Clonazepam	0.13	Bromazepam	4.03
Flunitrazepam	0.21	Desmethylchlordiazepoxide	4.65
Temazepam	0.30	Chlordiazepoxide	5.55
Oxazepam	0.36	Desoxychlordiazepoxide	7.02*
N-1-Hydroxyethylflurazepam	0.98	Medazepam	8.04*
N-1-Desalkylflurazepam	1.26	Flurazepam	10.86*
Triazolam	1.37	Midazolam	10.86*

* Tailing peak.

perchloric acid eluent, peak shapes were generally good at short retention times but gradually deteriorated with the compounds showing greater retention. Comparison of Tables II and III shows some differences in elution order between the two systems using different acid additives. As the HPLC columns used for measuring both sets of retention data contained the same batch of silica packing material the differences observed can be attributed to the eluent and not to column variations. Nevertheless, the elution properties of the two systems were very similar and the plot of k' values

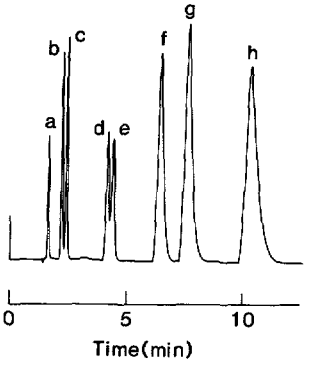

Fig. 5. Chromatography of benzodiazepines and metabolites on silica using the trifluoroacetic acid eluent (system D). Column, Spherisorb S5W, 5 μm (250 × 5 mm I.D.); eluent, methanol–water–trifluoroacetic acid (997:2:1, v/v/v); flow-rate, 2 ml/min; detection, 240 nm. Peaks: a = lorazepam; b = temazepam; c = oxazepam; d = N-1-desalkylflurazepam; e = nitrazepam; f = prazepam; g = diazepam; h = chlordiazepoxide.

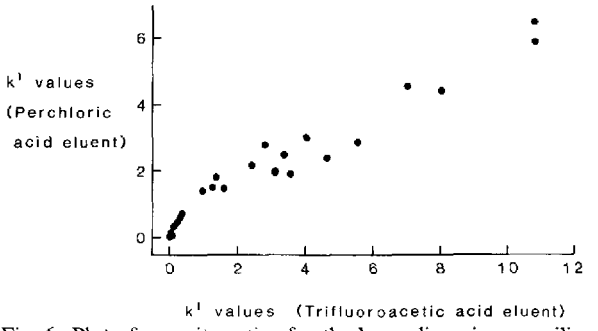

Fig. 6. Plot of capacity ratios for the benzodiazepines on silica using the perchloric acid eluent (system C) *versus* capacity ratios on silica using the trifluoroacetic acid eluent (system D). For details of the HPLC systems see Table IV.

TABLE IV

RETENTION DATA FOR BENZODIAZEPINES AND METABOLITES (ARRANGED IN ALPHABETICAL ORDER) ON FOUR HPLC SYSTEMS

System A: ODS-Hypersil using methanol–water–phosphate buffer (0.1 *M*) (55:25:20, v/v/v); system B: ODS-Hypersil using methanol–water–phosphate buffer (0.1 *M*) (70:10:20, v/v/v); system C: Spherisorb S5W using methanol (1000 ml) containing perchloric acid (100 μl); system D: Spherisorb S5W using methanol–water–trifluoroacetic acid (997:2:1, v/v/v).

Compound	*Capacity ratio (k')*			
	System A	*System B*	*System C*	*System D*
Parent drugs				
Alprazolam	4.70	–	2.79	2.79
Bromazepam	2.32	–	2.99	4.03
Chlordiazepoxide	6.41	–	2.87	5.55
Clobazam	3.91	–	0.03	0.02
Clonazepam	2.85	–	0.35	0.13
Clorazepic acid	1.17	–	2.00	3.12
Diazepam	9.47	2.29	2.49	3.35
Flunitrazepam	3.15	–	0.47	0.21
Flurazepam	–	3.19*	6.50*	10.86*
Ketazolam	12.81	2.45	0.04	0
Lorazepam	4.60	–	0.14	0.05
Lormetazepam	6.32	–	0.08	0.02
Medazepam	–	7.05*	4.44*	8.04*
Midazolam	9.75	2.10	5.90*	10.86*
Nitrazepam	2.96	–	1.49	1.60
Oxazepam	4.62	–	0.73	0.36
Prazepam	–	4.60	2.19	2.42
Temazepam	5.68	–	0.60	0.30
Triazolam	4.38	–	1.83	1.37
Metabolites				
7-Acetamidonitrazepam	0.68	–	1.93	3.58
7-Aminonitrazepam	0.46	–	0	0
Demoxepam	2.42	–	0.03	0.07
N-1-Desalkylflurazepam	5.19	–	1.52	1.26
Desmethylchlordiazepoxide	4.47	–	2.39	4.65
Desmethylclobazam	3.06	–	0.01	0
Desoxychlordiazepoxide	–	3.85	4.55*	7.02*
N-1-Hydroxyethylflurazepam	4.27	–	1.43	0.98
Nordazepam**	8.00	–	1.99	3.12

* Tailing peak.

** This metabolite of diazepam is also available as a drug in some countries.

(Fig. 6) demonstrates a high degree of correlation. Thus, the use of both systems to aid in the identification of an unknown benzodiazepine is unlikely to yield extra information.

In conclusion, four HPLC systems (two using ODS-silica and two using silica columns) have been used for the chromatography of a wide range of benzodiazepines and metabolites. All systems have used the standard packing materials which have been adopted by forensic science laboratories in the U.K. on which many HPLC systems for various drug analyses have already been developed. The retention properties of the benzodiazepines have been measured on the HPLC systems and Table IV presents all the data arranged as an alphabetical listing to facilitate rapid retrieval of information for a specific drug or metabolite. The data will prove to be useful when selecting an appropriate eluent for the analysis of a particular benzodiazepine as well as offering analytical systems suitable for the identification of unknown benzodiazepines.

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